



Expression of lysostaphin in milk of transgenic mice affects the growth of neonates

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Abstract

As an initial step towards enhancing mastitis resistance in dairy animals, we generated BLG-Lys transgenic mice that secrete lysostaphin, a potent antistaphylococcal protein, in their milk. In the current study, we continue our assessment of lysostaphin as a suitable antimicrobial protein for mastitis resistance and have investigated mammary gland development and function in three lines of transgenic mice. As the lines were propagated, there was a tendency for fewer BLG-Lys litters to survive to weaning (51% as compared to 90% for nontransgenic lines, $p = 0.080$). Nontransgenic pups fostered on dams from these three lines exhibited diminished growth rates during the first week of lactation. Rates of gain became comparable to pups on nontransgenic dams at later time points. Initial slow growth also resulted in decreased weaning weights for pups nursed by transgenic dams (15.35 ± 0.27 g) when compared to pups delivered and nursed by nontransgenic dams (18.61 ± 0.61 g; $p < 0.001$), but the effect was temporary, as similar weights were attained by adulthood. Milk yield at peak lactation was not different between BLG-Lys (0.79 ± 0.33 g) and nontransgenic (0.91 ± 0.38 g; $p = 0.166$) dams. Histological examination of the transgenic mammary glands during gestation revealed no differences when compared to control glands; however, at early lactational stages, the BLG-Lys glands exhibited less alveolar area than control glands and a delay in lobulo-alveolar maturation. The results clearly demonstrate reduced growth of neonates on BLG-Lys dams; whether the poor pup performance can be attributed to delayed mammary development or the gland development merely reflects reduced suckling stimuli from the pups remains to be determined.

Introduction

Mastitis, an inflammation of the mammary gland, is the most costly disease affecting dairy cows. Despite the implementation of mastitis control programs for decades, dairy farmers still incur serious economic losses as a result of reduced milk production and qual-

ity and the culling of infected animals. These losses exceed \$2 billion annually for the US dairy industry (Sordillo & Streicher, 2002). Antibiotics are widely used to combat mastitis; however, the cure rate for *Staphylococcus aureus* mastitis, which currently accounts for 15–30% of cases (Sutra & Poutrel, 1994; Waage et al., 1999), is often less than 15%.

The mammary gland, in addition to relying on the immune system, produces a number of milk proteins with antimicrobial properties, including lactoferrin, lysozyme, and lactoperoxidase (Reiter, 1978; Takahashi et al., 1992). Not surprisingly, researchers have attempted to combat mastitis by increasing expression of some of these proteins in the mammary

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gland. Initial attempts to express antibacterial proteins have resulted in the generation of transgenic mice that secrete human lysozyme (Maga et al., 1994), human lactoferrin (Platenburg et al., 1994), or bovine tracheal antimicrobial peptide (bTAP) (Yarus et al., 1996) in their milk. In identifying antimastitis candidate proteins that are amenable to the transgenic approach and suitable for the dairy industry, several criteria must be considered. The gene product cannot be detrimental to the general and reproductive health of the transgenic animal or its offspring. In addition, the protein cannot interfere with the development and functional integrity of the mammary gland. The protein should not compromise the nutritive quality or the manufacturing properties of the milk. Ideally, the candidate transgene product also should be either orally inactivated or destroyed by standard milk processing procedures.

In our first effort to enhance mastitis resistance in dairy animals through transgenesis, we have targeted a modified lysostaphin gene to the mammary gland of transgenic mice (Kerr et al., 2001). Lyso-staphin, a potent peptidase secreted by *Staphylococcus simulans*, specifically cleaves the pentaglycine bonds unique to the interpeptide bridge of the *S. aureus* cell wall (Schindler & Schuhardt, 1964). The effectiveness of bacterial lysostaphin in the prevention and control of *S. aureus* infection has been demonstrated in experimental models of food-borne illness (Cavadini et al., 1998), endocarditis (Patron et al., 1999) and endophthalmitis (Dajcs et al., 2001). Most importantly, the intramammary infusion of recombinant lysostaphin has been successfully used as a therapeutic technique for staphylococcal mastitis in both mouse (Bramley & Foster, 1990) and dairy animal (Oldham & Daley, 1991) models. We have demonstrated that transgenic mice expressing adequate concentrations of lysostaphin in milk are completely protected from *S. aureus* challenge (Kerr et al., 2001).

To assess more comprehensively the suitability of lysostaphin as an antimicrobial for mastitis resistance, we have investigated mammary gland development and function in three lines of transgenic mice. While milk yield, milk fat content and gene expression patterns appear normal in lysostaphin-expressing dams at peak lactation, neonates nursed by these dams exhibit diminished growth during the first few days of lactation. Histological examination of the BLG-Lys mammary glands in late gestation and early lactation indicate that, while the glands are indistinguishable from control glands during gestation, the achievement of full lactational competence after parturition

is delayed. Whether this diminished mammary development accounts for the impaired pup growth or the converse is true remains to be determined.

Materials and methods

Animal use and care

The generation of BLG-Lys transgenic mice was described previously (Kerr et al., 2001). Three expressing lines were included in the current studies. Each of the transgenic lines was propagated in the heterozygous state; that is, all the BLG-Lys animals described in these studies were the result of BLG-Lys transgenic \times B6SJL F1/J matings. Primiparous BLG-Lys transgenic females were utilized for all growth and milk production analyses; primiparous B6SJL F1/J females were used as controls. All procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved in advance by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee.

Growth performance and milk yield

To test whether transgenic dams influenced the growth performance of neonatal pups, nontransgenic pups were fostered on dams from the three BLG-Lys transgenic lines. For each BLG-Lys female bred in these analyses, four nontransgenic females were mated concurrently to ensure that sufficient numbers of age-matched nontransgenic pups would be available for fostering. Only nontransgenic females that delivered on the same day as the transgenic dams were included in the study. One day after parturition, one nontransgenic dam was designated randomly as a control dam. Six nontransgenic foster pups then were assigned randomly to each transgenic and control dam. The approach taken assured that no nontransgenic control dam reared any of her birth pups. Dams that did not successfully foster all six pups were excluded from the study. Litter weights were recorded at the time of pup assignment (designated as day 1 of lactation) and on days 6, 8, and 12 of lactation. On day 10, the weigh-suckle-weigh procedure was conducted as described below.

To evaluate the functional integrity of the mammary gland of BLG-Lys transgenic dams, milk yield was estimated by the weigh-suckle-weigh method

(Jara-Almonte & White, 1972) from the same dams included in the growth performance study. Transgenic and nontransgenic dams that did not retain six pups to day 10 of lactation were excluded from the analysis. To measure milk production, the litters were separated from the dams in the morning for 4 h. The litters were then weighed, replaced on the dams to nurse for 2 h, and weighed again after suckling. Milk yield was determined indirectly from the differences in the litter weights before and after suckling.

Weaning weights

Individual pup weights were measured upon separation from the dams at 30 days *post partum*. These animals included pups from the growth performance analyses, birth pups of the BLG-Lys and nontransgenic dams included in the analyses and other BLG-Lys pups and nontransgenic pups fostered on nontransgenic dams.

Milk fat determination

Mice were milked on day 10 of lactation following standard procedures (Maga & Murray, 1995). The percentage of milk fat was determined using the creatocrit method (Lucas et al., 1978). Briefly, approximately 100 μ l milk was centrifuged in a hematocrit capillary tube, resulting in the separation of cream as a discrete layer (Micro-capillary Centrifuge Model MB, IEC). The percentage of fat in the milk was then determined by the ratio of the length of cream to total milk in the column.

Mammary gland histology

Mice were euthanized at days 15 and 19 of gestation and days 3, 5 and 10 of lactation. Both abdominal mammary glands were harvested and weighed. The left gland was placed in Carnoy's fixative (chloroform:ethanol:glacial acetic acid; 6:3:1), washed in 70% ethanol, and encased in a polypropylene-embedding cassette. Fixed tissues were embedded in paraffin, cut in 5 μ m sections, and stained with hematoxylin and eosin. Five random images were collected for each section, two sections per gland, and alveolar area was determined as a percentage of total area using Scion Image, version 4.0.2 ('NIH Image' for the PC, Scion Corporation, <http://www.scioncorp.com/>).

Evaluation of transgene/milk protein expression by northern blot analysis

Total RNA was isolated from the right abdominal mammary glands using a guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987) and quantified on a spectrophotometer. Equal amounts of RNA (20 μ g) were loaded in each lane. Northern blots were simultaneously probed with 32 P-labeled Gln^{125,232}-lysostaphin, β -casein, and G3PDH cDNAs and then analyzed with a phosphorimager (STORM, Molecular Dynamics). Both lysostaphin and β -casein expression were normalized to G3PDH expression levels.

Statistical analysis

Values are expressed as least square means \pm SEM. Litter survival was compared by Chi-square analysis. All other comparisons, such as litter weights, growth rates, individual weaning weights, milk production, milk fat, mammary gland weights, and alveolar area were evaluated with the General Linear Model module of SPSS, version 8 software (SPSS, Inc.). Percentages and ratios were ArcSin transformed prior to analysis. The body weight of the dam and the number of pups *in utero* (G15 and G19) or delivered (L3 and L5) served as a covariate in the statistical analysis of mammary gland weights. When initial ANOVA failed to detect differences among lines, observations for lines were pooled and compared to the nontransgenic group in a second analysis.

Results

Previously, we described the generation of transgenic mice carrying a bioactive variant of lysostaphin, Gln^{125,232}-lysostaphin, expressed in the mammary gland under control of the ovine β -lactoglobulin cassette (BLG-Lys; Kerr et al., 2001). Lines 535, CS and BS, expressing lysostaphin in their milk at concentrations of 39.1 ± 4.9 μ g/ml, 31.3 ± 5.2 μ g/ml, and 30.3 ± 4.6 μ g/ml respectively, were expanded for further analysis. As these transgenic lines were being propagated, there were indications that the pups were not thriving. When compared to other transgenic lines (MLC-Pro4; Yang et al., 2001) and nontransgenic mice bred concurrently, there was a tendency for fewer BLG-Lys litters to survive to weaning (MLC-Pro4: 71%, $n = 49$; nontransgenic: 90%, $n = 41$; BLG-Lys: 51%, $n = 75$; $p = 0.080$). The reduced

survival was not the result of smaller transgenic litters as litter size at birth did not differ between the BLG-Lys (7.2 ± 0.3 pups/litter, $n = 47$) and nontransgenic (7.7 ± 0.5 pups/litter, $n = 22$; $p = 0.144$) lines. We hypothesized that poor performance of the BLG-Lys offspring could have been caused by insufficient milk production by the transgenic dams, reduced nutritive quality of the milk produced, a detrimental effect of the transgene product in the milk, or a direct influence of transgene expression in the pups.

In order to distinguish whether decreased survival was attributable to the performance of the dams or the pups, nontransgenic pups were randomly assigned to dams from the three BLG-Lys lines and to nontransgenic dams. Litter weights were then measured from day 1 to day 12 of lactation, after which time pups begin to eat solid food. Even at a gross level, it became apparent that the litters fostered by BLG-Lys transgenic dams were not growing as well as the litters fostered by nontransgenic control dams. Although litters did not differ in weight at the start of the growth trial (day 1 of lactation, Figure 1), by day 6, the litters suckled by 535 and CS dams weighed significantly less ($535: 18.58 \pm 1.37$ g; $CS: 19.02 \pm 1.62$ g) than the litters on nontransgenic dams (23.88 ± 0.74 g; $p = 0.006$). By day 10, litters raised by dams of all three BLG-Lys lines weighed less than the litters on nontransgenic dams ($p = 0.001$). The litter weights of

pups on dams from the three transgenic lines did not differ from each other at day 12 ($535: 33.83 \pm 1.62$ g; $CS: 34.01 \pm 1.91$ g; $BS: 37.08 \pm 1.51$ g; $p = 0.150$), but remained different from litters nursed by nontransgenic dams (40.80 ± 0.87 g; $p = 0.039$).

The growth rate of litters nursed on BLG-Lys dams was clearly compromised by day 6 of lactation (Figure 1). It appeared that the poor performance of these litters could be accounted for by minimal growth during the first few days of life. This conclusion was reached by assuming that the rate of growth between day 1 and day 6 was similar to the rate between day 6 and day 12 and extrapolation of the growth curves toward zero (insert, Figure 1). Indeed, when litter weights were measured at day 3 of lactation, a difference could be detected between litters on transgenic or nontransgenic dams (BLG-Lys: 8.87 ± 0.53 g, $n = 6$; nontransgenic: 11.27 ± 0.64 g, $n = 4$; $p = 0.020$). After day 6 of lactation, the growth rates measured over 2 day increments were not different between litters on transgenic and control dams, again suggesting the factors influencing growth of the litters were confined to the early lactation period. However, overall growth rate between day 6 and day 12 differed by about 10% for pups on transgenic and nontransgenic dams (2.53 ± 0.06 v.s. 2.82 ± 0.05 g/day respectively, $p = 0.001$). This observation indicated that the detrimental effects were not completely resolved by day 12 of lactation. As all the pups included in this growth study were nontransgenic, the observations demonstrated that diminished litter growth was not caused by transgene expression in the pups.

Weaning weights also confirmed that transgenic status of the pups was not an influential factor in growth performance. At weaning, the individual weights of transgenic and nontransgenic pups raised by nontransgenic dams did not differ significantly (Table 1). In addition, no difference was detected between the adult weights of transgenic and nontransgenic females suckled by nontransgenic dams (Table 1). Nontransgenic pups fostered by BLG-Lys dams, however, weighed less on the day of weaning than nontransgenic pups fostered by nontransgenic dams (Table 1). The diminished weight at weaning, however, did not compromise the ability of pups nursed by BLG-Lys dams to attain adult stature. Transgenic BLG-Lys females raised on BLG-Lys dams achieved the same weight at adulthood (26.3 ± 0.7 g, $n = 16$) as nontransgenic females raised on nontransgenic dams (28.3 ± 0.7 g, $n = 19$; $p = 0.163$).

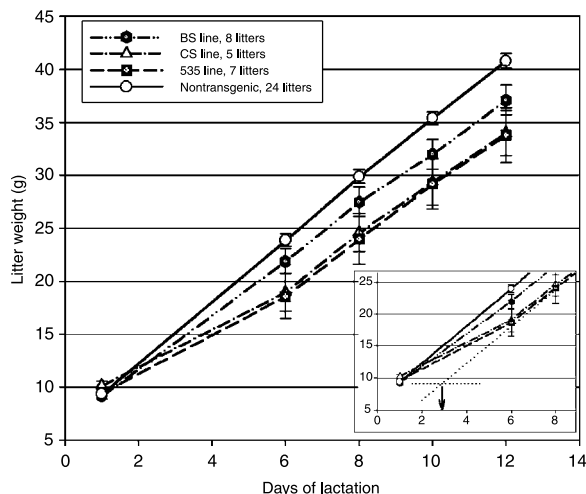


Figure 1. Litter weights of six nontransgenic pups fostered on dams of BLG-Lys transgenic lines 535, CS, and BS and nontransgenic dams through day 12 of lactation. Insert: Assuming growth rate (slope of curves) is approximately the same between days 1 and 6 and days 6 and 12, extrapolation of curves of the transgenic dams suggests growth rate of pups was minimal during the first few days of life.

Table 1. Mammary gland function parameters at peak lactation (Means \pm SEM)

Item	BLG-Lys transgenics	Nontransgenics
Pup weight at weaning (TG v.s. nonTG pups, g) ^a	14.91 \pm 0.36, <i>n</i> = 48	15.41 \pm 0.37, <i>n</i> = 62
Pup weight at weaning (TG v.s. nonTG dams, g) ^b	15.35 \pm 0.27, <i>n</i> = 79	18.61 \pm 0.61, <i>n</i> = 15
Adult female weight (g) ^c	27.10 \pm 0.70, <i>n</i> = 17	28.25 \pm 0.68, <i>n</i> = 19
Milk yield (g) ^d	0.79 \pm 0.33, <i>n</i> = 32	0.91 \pm 0.38, <i>n</i> = 37
Milk fat (%) ^e	27 \pm 3, <i>n</i> = 10	29 \pm 4, <i>n</i> = 7
β -Casein expression (pixel intensity) ^f	224 \pm 17, <i>n</i> = 6	241 \pm 30, <i>n</i> = 2

^a BLG-Lys or nontransgenic pups fostered by nontransgenic dams did not differ in weight at weaning.

^b Nontransgenic pups fostered by transgenic dams weighed less than nontransgenic pups fostered by nontransgenic dams at weaning, *p* < 0.001.

^c Adult body weight of BLG-Lys and nontransgenic females fostered by nontransgenic dams did not differ.

^d Milk yield of BLG-Lys and nontransgenic dams did not differ at day 10 of lactation.

^e Percentage of milk fat did not differ between transgenic and nontransgenic dams at day 10 of lactation.

^f β -Casein expression as determined by northern analysis (normalized to G3PDH expression) did not differ at day 10 of lactation.

Impaired mammary gland function in the transgenic dams would account for poor growth performance. Measuring milk production at peak lactation is a standard method of assessing mammary gland function (Jara-Almonte & White, 1972); therefore, weigh-suckle-weigh experiments were performed on day 10 of lactation. A tendency was observed for fewer BLG-Lys transgenic dams to retain all six pups to day 10 (535: 71%, *n* = 14; CS: 64%, *n* = 14; BS: 72%, *n* = 18; nontransgenic: 90%, *n* = 41; *p* = 0.174). The results of the weigh-suckle-weigh experiment, however, failed to detect a difference in milk consumption between litters on

transgenic and nontransgenic dams (Table 1), suggesting that mammary gland function of the dams was similar. As no difference could be detected in the quantity of milk produced by transgenic and nontransgenic dams, the nutritive quality, as assessed by fat content (Hamosh, 1979), was evaluated. Milk collected from other transgenic and nontransgenic dams at this stage of lactation had comparable fat content (Table 1).

As the growth curves suggested the primary problem occurred early in lactation and the glands appeared to function normally at peak lactation, the possibility of delayed mammary development was

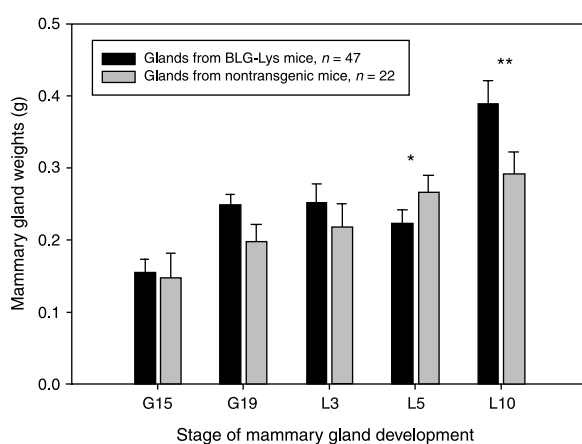


Figure 2. Weights of mammary glands from BLG-Lys transgenic and nontransgenic mice collected at days 15 and 19 of gestation and days 3, 5 and 10 of lactation. Least square means were adjusted to account for influence of dam body weight and litter size. Glands from transgenic and nontransgenic mice differed in weight on day 5 (**p* = 0.020) and day 10 (***p* = 0.001) of lactation.

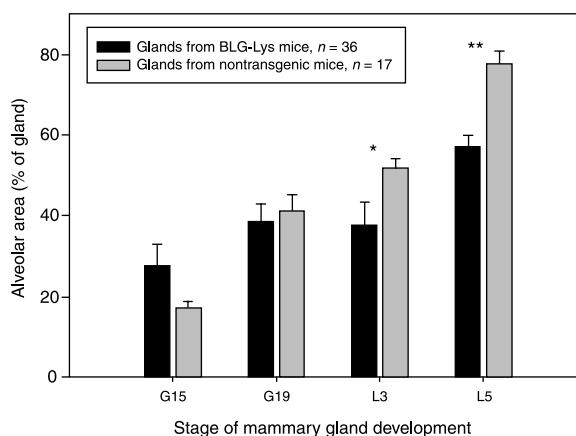


Figure 3. Alveolar area of mammary glands from BLG-Lys transgenic and nontransgenic mice collected at days 15 and 19 of gestation and days 3 and 5 of lactation. Mean alveolar area was determined for five random images per histological section, two sections per gland. Alveolar area was smaller in glands from transgenic females on day 5 (***p* = 0.001) of lactation, but not on day 3 (**p* = 0.054).

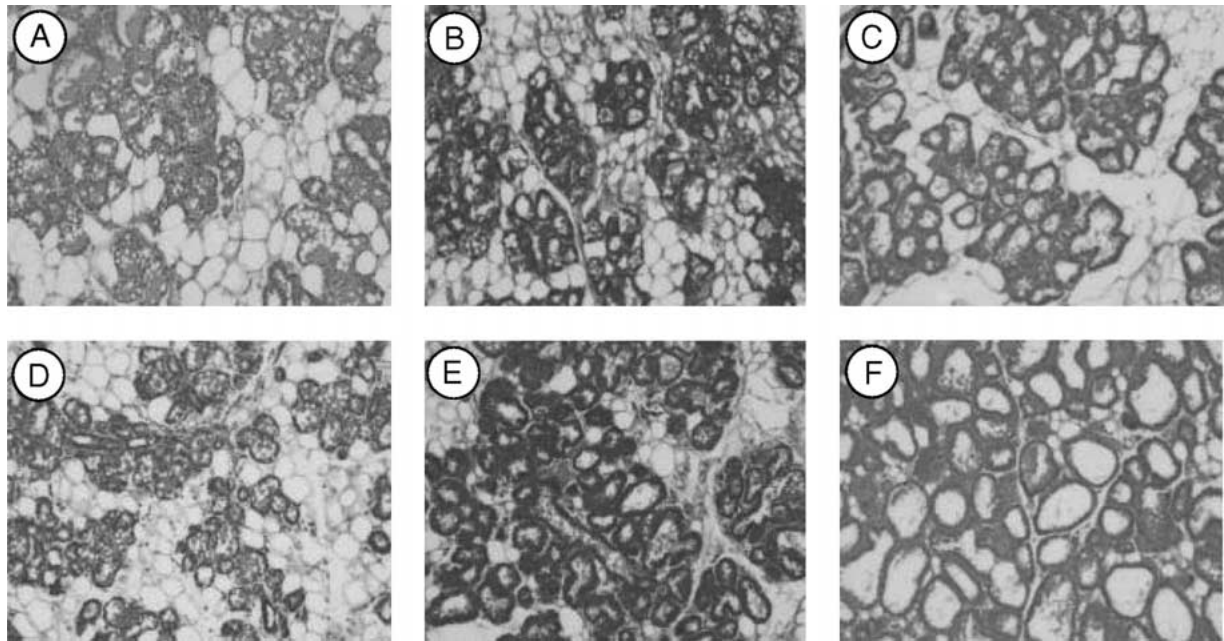


Figure 4. Histological analysis of mammary gland development in BLG-Lys transgenic and nontransgenic mice. Panels A, B and C represent glands from BLG-Lys transgenic mice at day 19 of gestation, day 3 of lactation and day 5 of lactation, respectively. Panels D, E and F represent glands from nontransgenic mice at day 19 of gestation, day 3 of lactation and day 5 of lactation, respectively. Magnification: 200 \times .

investigated. The weights and morphology of abdominal mammary glands collected on days 15 and 19 of gestation (G15 and G19), and days 3, 5 and 10 of lactation (L3, L5 and L10) were evaluated. Both body weight of the dam and litter size had a significant influence on mammary gland weight, therefore, these parameters were included as cofactors in the analysis. The glands of the BLG-Lys and nontransgenic dams did not differ in weight through gestation and day 3 of lactation, but the glands of the BLG-Lys dams were significantly lighter at day 5 of lactation (Figure 2). By day 10 of lactation, the glands of the transgenic dams rebounded to become heavier than the glands of nontransgenic dams (Figure 2).

In order to determine if secretory capacity differed between the BLG-Lys and nontransgenic glands, the development of the lobulo-alveolar compartment was examined by histology. A quantitative analysis of the percentage of alveolar area in the glands (Figure 3) detected no differences between transgenic and control glands during gestation. By day 3 of lactation, however, there was a tendency for the BLG-Lys transgenic dams to have less alveolar area per gland when compared to nontransgenic dams ($37.7 \pm 5.8\%$ v.s. $51.9 \pm 2.4\%$, $p = 0.054$) and by day 5 of lacta-

tion, there was a clear difference in alveolar area ($56.9 \pm 3.1\%$ v.s. $78.1 \pm 3.0\%$, respectively, $p = 0.001$).

Histological examination of BLG-Lys and nontransgenic glands confirmed the quantitative measurements. Differences in the lobulo-alveolar compartment at day 19 of gestation were not apparent (Figure 4: panels A, and D). Cytoplasmic vacuolation and secretion was well established in both the BLG-Lys and nontransgenic glands. At day 3 of lactation, however, the BLG-Lys transgenic glands demonstrated limited alveolar distension and secretion (Figure 4: panel B), while the nontransgenic mammary glands showed symmetrical expansion of the alveoli (Figure 4: panel E). Notably, the transgenic glands at day 3 of lactation showed minimal change from late gestation (Figure 4: panels A, and B). By day 5 of lactation, the alveoli of the BLG-Lys transgenic mice had expanded to a comparable extent as control alveoli at day 3 of lactation (Figure 4: panels C, and E, Figure 3). In contrast, the lobulo-alveolar compartment of the nontransgenic glands comprised about 80% of the total glandular area at day 5 (Figure 4: panel F, Figure 3).

Northern analyses demonstrated that the lyso-staphin transgene was moderately expressed by day

15 of gestation in the BLG-Lys glands (data not shown). The expression patterns of β -casein in the BLG-Lys and nontransgenic glands did not differ during gestation or peak lactation (data not shown and Table 1).

Discussion

In this study, we have demonstrated that expression of an antimicrobial protein in the mammary gland of BLG-Lys transgenic dams affects the growth performance of neonates suckling on these dams. The diminished growth of nontransgenic pups fostered on BLG-Lys transgenic dams (Figure 1) isolated the problem to transgene expression in the dam. Functional heterologous proteins have been produced in the mammary gland at gram/liter concentrations without ill effect (Lee and de Boer, 1994; Maga and Murray, 1995; Clark, 1996; Lonnerdal, 1996), but precocious expression of milk proteins has been associated with impaired alveolar development (Burdon et al., 1991). Previous studies in which antimicrobial proteins were expressed in the mammary gland (Maga et al., 1994; Platenburg et al., 1994; Yarus et al., 1996) did not report diminished pup growth.

The data presented here demonstrate that the BLG-Lys glands were not compromised at peak lactation as measured by milk yield, milk fat content, and β -casein expression (Table 1). The diminished growth of pups fostered on BLG-Lys dams seems attributable, rather, to a delay in achieving lactational competence. Mammary gland development proceeded normally throughout the gestational period in BLG-Lys dams; no differences were detected in gland weight, alveolar area or structure when compared to control dams (Figures 2–4). After parturition, however, significant differences were observed in gland weight and alveolar area. At days 3 and 5 of lactation, the 14 and 21% differences in alveolar area of the BLG-Lys dams compared to control dams reflected the weight differences of litters fostered by these respective dams. Histological examination suggested that the BLG-Lys glands functionally differentiated, but were delayed in reaching full secretory capacity.

Two general mechanisms could account for the apparent delay in *post partum* mammary development. While systemic hormones signal alveolar proliferation and functional differentiation during pregnancy, an elaborate network of systemic hormones and local

growth modulators, as well as the initiation of suckling and milk withdrawal, are responsible for further *post partum* proliferation and the full establishment of lactation. The expression of lysostaphin in the mammary gland may exert a direct effect on gland development and maturation. Recent documentation of transgene expression that results in retardation of alveolar development includes the expression of a dominant-negative BRCA1 (Brown et al., 2002) and the only zinc finger (OZF) (Besnard et al., 2002). No effect on pup growth was reported for the trBRCA1 dams however, while homozygous OZF dams could not sustain full growth of their pups. In both reports, the 2–3 day delay in development was evident during gestation. We were unable to detect differences in mammary development at the gestational stages analyzed.

Biochemical studies have demonstrated that lysostaphin can participate in elastin degradation with an activity that is distinct from its staphylytic activity (Park et al., 1995). Elastin is an important component of the extracellular matrix (ECM). The signals required for maintenance of function during lactation arise primarily from the ECM (Howlett & Bissell, 1993). Transgenic and knock-out experiments that disrupt components of the ECM have resulted in seriously compromised mammary development and lactational failure (Hathaway & Shur, 1996; Lund et al., 2000; Nemir et al., 2000). While the observed phenotype in the BLG-Lys mammary glands is not as pronounced as in these examples, it is possible that expression of lysostaphin interferes with local signaling between the epithelium and ECM. This effect may be limited because *in vitro* and *in vivo* experiments have demonstrated that elastin mRNA and protein are up-regulated in response to elastolytic activity (Rich et al., 2003).

Alternatively, expression of lysostaphin may impact mammary gland development indirectly by influencing the suckling behavior of the pups. At parturition, the gland is committed to lactation, but alveolar expansion and development continue during the *post partum* period to meet the growing needs of the neonates. If a dam loses her litter or the teats are sealed experimentally, milk will accumulate in the alveolar lumens and the gland will initiate involution (Li et al., 1997; Marti et al., 1997). Lysostaphin in the milk of transgenic dams may reduce suckling by simply making the milk less palatable. Clearly, if this is the case, the pups on BLG-Lys dams acclimate to the taste, as their milk consumption is

comparable to pups on nontransgenic dams at peak lactation

Another intriguing possibility is an effect of lysostaphin on the establishment of the intestinal microflora of the neonates. The microflora is acquired postnatally and composed of a diverse population of bacterial cells that can perform beneficial functions for the host, such as promotion of gut maturation and integrity, stimulation of peristalsis, and maintenance of intestinal immune homeostasis. A significant reduction in the absorptive capacity (Heyman et al., 1986) and enteroendocrine cell number (McCullogh et al., 1998) of the small intestine has been documented in germ-free mice. In addition, the length of the small intestine is shorter in specific-pathogen-free (SPF) swine than in conventional swine (Mochizuki & Makita, 1998). It is conceivable that expression of lysostaphin, an anti-staphylococcal peptide, in the milk of BLG-Lys transgenic dams, may disrupt early settlement of *Staphylococci* in the gut and affect the normal installation of microflora. Lactoferrin, another antimicrobial protein found in milk, has been shown to suppress the proliferation of various bacteria (Teraguchi et al., 1994, 1995b) and bacterial translocation (Teraguchi et al., 1995a) in the intestines of juvenile SPF mice fed bovine milk. Poor development of the neonatal digestive tract would, in itself, result in smaller pups. In addition, pups that are not thriving may exhibit diminished suckling behavior, thereby accounting for the observed *post partum* development in the BLG-Lys gland.

In conclusion, we have demonstrated that lysostaphin expression results in diminished *post partum* development of the mammary gland and poor initial growth of pups. This effect is isolated to the early lactation period and does not have long-term consequences for the growth of neonates nursed on BLG-Lys transgenic dams. It is not possible to distinguish at this time if the effect of lysostaphin on the mammary gland is direct or indirect. We must further confirm that lysostaphin does not compromise the nutritive quality or the manufacturing properties of bovine milk and that its antimicrobial properties are orally inactivated or destroyed by pasteurization. Should lysostaphin satisfactorily meet these criteria, we can consider the possibility that the concerns raised by the growth performance in the mouse lines may be specific to the model system. If such is the case, expression of lysostaphin in the mammary gland of dairy cows may become a useful strategy for combating mastitis.

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